Analysis of phosphorylation of pRB and its regulatory proteins in breast cancer

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Abstract

Aim—In order to study the role of retinoblastoma protein (pRB) in breast cancer, the phosphorylation of pRB and the expression of its related proteins—such as cyclin E, cyclin dependent kinase 2 (Cdk2), and p21/Cdk interacting protein 1 (Cip1)—were examined in 30 breast cancers in which pRB overexpression was confirmed immunohistochemically.

Methods—The phosphorylation of pRB for 30 tumours was investigated with western blotting. The expression of pRB, Cdk2/Cdc2, cyclin E, and p21/Cip1 was identified by immunohistochemistry and western blotting.

Results—The expression of pRB was confirmed in 52 of 70 tumours (74%) by immunostaining. Western blotting for pRB showed that 25 of 30 representative cancers (83%) were underphosphorylated, while only five tumours showed the hyperphosphorylated form of pRB. However, cyclin E and Cdk2—which promote phosphorylation of pRB—were expressed in all tumours. On the other hand p21/Cip1, a Cdk2 inhibitor, was expressed in 18 of 25 tumours with underphosphorylated pRB, while four of the five tumours with hyperphosphorylated pRB showed no expression of p21/Cip1. Examination of the relation between pRB phosphorylation and clinicopathological variables showed that the underphosphorylated group was characterised by low risk of lymph node metastasis ($p < 0.01$).

Conclusions—The phosphorylation of pRB appears to be regulated mainly by p21/Cip1 through the suppression of cyclin E and Cdk2 in breast cancer. The underphosphorylated form of pRB may be useful as a prognostic factor.

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Keywords: pRB; Cdk2; cyclin E; p21/Cip1; breast cancer

The Rb gene is a typical oncosuppressor gene that is involved in the formation of hereditary or sporadic retinoblastoma.¹ Loss or structural abnormalities of this gene have been found in several other cancers, including breast cancer.²⁻⁶ The Rb gene encodes a nuclear protein (pRB) with a molecular weight of 110 kDa,⁷ which was found to be a negative regulator of the cell cycle. Loss of this function

of pRB may be responsible for uncontrolled cell cycle progression, ultimately leading to carcinogenesis or tumour progression. This function of pRB is thought to be regulated by phosphorylation. pRB has been shown to be phosphorylated in a cell cycle dependent manner, being underphosphorylated in the G0-G1 phase and highly phosphorylated during the G1 to S boundary.⁸ This may be mediated by binding of pRB to transcription factor E2F: only underphosphorylated pRB can form a complex with E2F to block the activity of E2F, which activates the transcription of genes that encode the proteins required for the progression to the S phase.⁹⁻¹⁰ These data suggest that pRB is active in its underphosphorylated and inactive in its hyperphosphorylated form.

The phosphorylation of pRB has been shown to be mediated by a complex of cyclin dependent kinase 2 (Cdk2) and cyclin E in higher eukaryotes.¹¹⁻¹⁴ On the other hand, p21/Cip1 binds to Cdk2 and blocks the function of this complex,¹⁵ leading to inhibition of phosphorylation of pRB.

The expression of pRB has been studied immunohistochemically by several investigators. Sawan *et al* found that pRB was expressed in 78% of 197 breast cancers,¹⁶ and Trudel *et al* in 81% of 100 breast cancers.¹⁷ Our results were largely consistent with these findings. However, an immunohistochemical study with the monoclonal antibody for pRB cannot determine whether pRB is phosphorylated. To clarify whether pRB is functional or not, western blotting for pRB is required. In this paper, we report on the phosphorylation of pRB identified by western blotting in 30 breast cancers which were confirmed by immunostaining as expressing pRB. The regulatory proteins of pRB were also studied.

Methods

CELL LINES AND TISSUE SAMPLES

MCF-7, a breast cancer cell line, was purchased from the Japanese Cancer Research Resources Bank. Tissue samples were obtained from 30 patients undergoing surgery for breast cancer between 1990 and 1994. The patients' ages ranged from 33 to 82 years (mean 52.6 years). Histological grading was done in accordance with Elston's method.¹⁸⁻¹⁹ Cytosolic oestrogen receptor concentrations were determined with the EIA (enzyme immunoassay) technique, and values of more than 5 fmol/mg tissue protein were judged to be positive.

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ANTIBODIES

Anti-pRB monoclonal antibody PMG3-245 was purchased from Pharmingen (San Diego, California, USA). This antibody reacts with an epitope located between amino acids 300–380 of authentic pRB. Anti-Cdk2/Cdc2 monoclonal antibody 5F6 was obtained from Medical and Biological Laboratories (Nagoya, Japan). This antibody was raised against a synthetic peptide which includes the PSTAIRE motif²⁰ common to both Cdk2/Cdc2, so that this 5F6 recognises both Cdk2 and Cdc2. Anti-Cdk2, purchased from Upstate Biotechnology (Lake Placid, New York, USA), reacts specifically with an epitope within the C-terminal domain (residues 287–298) of human Cdk2. This antibody cannot be used for immunostaining with paraffin sections but only for western blotting. Cyclin E antibody, purchased from Upstate Biotechnology, reacts with an epitope contained within the N-terminal domain (residues 1–128) of human cyclin E. Anti-p21/Cip1 monoclonal p21/Cip1 antibody was purchased from Oncogene Science (Cambridge, Massachusetts, USA).

WESTERN BLOT ANALYSIS

A 100 µg sample of tissue was lysed with 1.0 ml of 0.1% SDS RIPA buffer (1% NP-40, 0.1% sodium deoxycholate, 0.15% NaCl, 50 mM Tris-HCl pH 7.5, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1% sodium dodecyl sulphate (SDS)) with the aid of a homogeniser. The lysate was centrifuged at 15 000 ×g at 4°C for 20 minutes and the supernatant collected. The protein concentration was determined by the Bradford protein assay (Bio-Rad, California, USA), followed by addition of ×5 SDS stop solution (1 M Tris-HCl pH 6.8, 15% SDS, 10% glycerol, 0.2% Bromophenol Blue (BPB), 5% 2-mercaptoethanol) to the supernatant until the final concentration of the lysate was adjusted to 2% SDS. For direct western blotting of the entire lysate, each lane was loaded with 75 µl of lysate containing 100 µg protein. Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis, after which the gel was soaked in blotting buffer (39 mM glycine, 48 mM Tris, 20% (vol/vol) methanol, and 0.02% SDS), shaken, and electroblotted onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts, USA). The Immobilon-P filter was then submerged in a blocking solution (5% non-fat dry milk) and incubated overnight at 4°C. After three washings with 1 × TBS containing 0.2% Tween 20 (TTBS) (Sigma, St Louis, Missouri, USA), the membrane was incubated with monoclonal antibody RB and p21/Cip1, or polyclonal antibody cyclin E and Cdk2 at a final concentration of 5 µg/ml at room temperature for one hour. The membrane was then incubated with anti-mouse IgG antibody or with anti-rabbit IgG antibody (Promega, Madison, Wisconsin, USA) at a dilution of 1:3000 at room temperature for one hour. The filters were washed with TTBS and probed with the ProtoBlot NBT and BCIP colour development system (Promega) according to the manufacturer's directions.

IMMUNOHISTOCHEMISTRY

Of the 30 representative samples of which the expression of pRB was confirmed by immunostaining, serial sections of 4 µm thickness were prepared and analysed for Cdk2/Cdc2, cyclin E, and p21/Cip1. Tissue sections were prepared from paraffin embedded breast tumour specimens, deparaffined in xylene, and dehydrated through a graded ethanol series. After quenching the endogenous peroxidase activity for 20 minutes in 0.1% (wt/vol) sodium azide containing 0.3% (vol/vol) hydrogen peroxide, non-specific binding was blocked by treatment with 10% (vol/vol) normal rabbit serum for 15 minutes. Antibodies 5F6, cyclin E, and p21/Cip1 were applied to each section at a dilution of 1:100, 1:250, and 1:50, respectively, after which the sections were incubated overnight at 4°C in a moist chamber. After washing in 0.05 mol/l phosphate buffered saline (PBS), biotinylated rabbit anti-mouse immunoglobulin (Histofine SAB-PO kit, Nichirei, Tokyo, Japan) for Cdk2/Cdc2 and p21/Cip1 and biotinylated goat antirabbit immunoglobulin for cyclin E were applied, and the slides incubated for 20 minutes at room temperature. After thorough washing in PBS, peroxidase conjugated streptavidin (Histofine SAB-PO kit) was applied, followed by incubation for 20 minutes. After the excess complexes had been washed, the localisations of Cdk2/Cdc2, cyclin E, and p21/Cip1 were visualised by incubating the slides for three to four minutes in 0.05 mol/l Tris-HCl (pH 7.6) containing both 0.02% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride and 0.03% (vol/vol) hydrogen peroxide. Negative controls employing normal mouse and rabbit serum were also incubated in conjunction with each staining procedure. A tumour with more than 10% of its cancer cells staining positively was scored positive, and one with less than 10% was scored negative.

STATISTICAL ANALYSIS

Contingency tables were used to determine the relation between the state of pRB phosphorylation and clinicopathological features of breast cancer. Statistical correlation was determined by χ^2 ; p values < 0.05 were considered to be statistically significant.

Results

WESTERN BLOTTING

pRB migrates as multiple bands as a result of varying degrees of phosphorylation. The molecular weight of the underphosphorylated form of pRB has been shown to be located at 110 kDa, while phosphorylated and highly phosphorylated pRB are represented by a cluster of bands around 110–116 kDa.^{21,22} Western blotting for pRB showed that 25 of the 30 tumour samples (83%) had a single band at 110 kDa, corresponding to underphosphorylated pRB. On the other hand, only five tumours and the MCF-7 cell line had a slowly migrating band around 110–116 kDa, corresponding to hyperphosphorylated pRB. In normal breast tissues, protein concentrations were either much lower or undetectable (fig 1).

Figure 1 Western blotting using a monoclonal antibody pRB of extracts from cultured cells and in matched pairs of normal breast glands and breast cancer tissues. N, normal tissue; T, tumour tissue; HL-60, promyeloid leukaemia cell line and positive control of hyperphosphorylated pRB; CV-1, monkey kidney fibroblast cells and positive control of underphosphorylated pRB. T1 is a representative case of underphosphorylated pRB and T2 is a representative case of hyperphosphorylated pRB.

Figure 2 Western blot analysis for Cdk2 and cyclin E.

Figure 3 Western blotting for P21/Cip1. Case T2 with hyperphosphorylated pRB lacked the expression of Cip1.

Examination of the relation between the phosphorylation of pRB and some clinico-pathological variables showed that tumours with underphosphorylated pRB were less likely to have lymph node metastasis ($p < 0.01$) (table 1).

Cdk2 was strongly expressed at 33 kDa in all tumour samples examined, while no signal was detectable in normal tissue samples (fig 2). Cyclin E, which showed a major signal at 52 kDa, was much more prevalent in all tumour samples examined than in normal tissue samples (fig 2).

The expression of p21/Cip1 was seen at 21 kDa, and was greater in tumour tissues than in normal tissues (fig 3).

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The population of cells positive for pRB, Cdk2/Cdc2, cyclin E, and p21/Cip1 in normal ducts and glands was considerably lower than in cancer regions. Thus these observations were largely consistent with those for western blotting (figs 4 and 5). Cyclin E and Cdk2/Cdc2 proteins were positively stained in the nuclei and cytoplasm of almost all cancer cells examined (fig 5).

The expression of pRB was confirmed in the nuclei of 52 of the 70 tumours (74%), while p21/Cip1 was expressed in the nuclei of 19 of the 30 tumours (63%) (fig 5). Moreover, 18 of the 19 Cip1 positive tumours showed underphosphorylated pRB. On the other hand, 18 of the 25 tumours with underphosphorylated pRB expressed p21/Cip1, while four of the five tumours with hyperphosphorylated pRB lacked p21/Cip1 expression (tables 2 and 3). A statistically significant relation was thus identified between pRB phosphorylation and p21/Cip1 expression ($p < 0.05$) (table 1). However, no significant association was found between the prognostic indices and the data for Cdk2/Cdc2 and cyclin E expression (data not shown).

Discussion

pRB is one of the key proteins involved in cell cycle control by regulating the G1-S transition. Structural abnormalities of the Rb gene have been found in 20–30% of breast cancers.^{4,6} In these cases, the production of pRB may have

Table 1 Relation between pRB phosphorylation and clinicopathological variables

	pRB	
	Underphosphorylated	Hyperphosphorylated
Histological grade		
I	7	0
II	14	3
III	4	2
Lymph node metastasis		
No	16	0*
Yes	9	5*
Oestrogen receptor status		
Negative	7	3
Positive	14	2
Stage		
I	5	0
II	14	2
III	6	3
Total	25 (83%)	5 (17%)

* $p < 0.01$.

Figure 4 (A) Immunostaining of pRB and (B) of p21/Cip1 in normal mammary glands. Only a few positive cells are observed in the nuclei, (A) $\times 100$; (B) $\times 240$. (C) Immunostaining of Cdk2 and (D) of cyclin E in normal mammary glands. A few immunoreactive cells are observed in the nuclei and cytoplasm of normal cells ($\times 100$).

been reduced, rendering the cell cycle uncontrollable. This would then be responsible for the genesis or rapid progression of the cancer. On the other hand, recent studies suggest that the dysfunction of some proteins in the regulation of pRB could also lead to uncontrolled cell growth,^{23–25} even if there were no alterations in the Rb gene itself. These findings indicate that reductions in either the amount or the function of pRB can result in abnormal cell growth.

Several investigators have reported a high frequency of immunohistochemical pRB expression in breast cancer.^{16, 17} We also examined the expression of pRB in 70 breast cancers with results largely consistent with those of the other studies. However, immunohistochemistry cannot determine whether pRB is functional. To clarify the role of pRB in breast cancer, we used western blotting to examine its phosphorylation in 30 tumours representative of the above mentioned breast cancers. It was found that 25 tumours showed the underphosphorylated form of pRB, while all of them showed overexpression of pRB in the immunohistochemical study. In other words, pRB was active in most breast cancers, and seems to function negatively in their cell proliferation.

It then became necessary to determine what factor keeps pRB underphosphorylated in these tumours. The phosphorylation of pRB in higher eukaryotes has been shown to be regulated by Cdk2,^{11, 12} while the function of Cdk2 was found to be activated by cyclin E^{26–28} and inhibited by p21/Cip1.¹⁵ Therefore, we examined the expression of cyclin E, Cdk2, and p21/Cip1 in the

Table 2 pRB phosphorylation and the expression of cyclin E, Cdk2 and p21/Cip1

Case	pRB We	p21/Cip1		Cdk2		Cyclin E	
		Im	We	Im	We	Im	We
1	Un	+	+	+	+	+	+
2	Un	+	ND	+	ND	+	ND
3	Un	+	+	+	+	+	+
4	Un	+	+	+	+	+	+
5	Un	+	ND	+	ND	+	ND
6	Un	+	+	+	+	+	+
7	Un	+	+	+	+	+	+
8	Un	+	+	+	+	+	+
9	Un	+	ND	+	+	+	ND
10	Un	+	+	+	+	+	+
11	Un	+	+	+	+	+	+
12	Un	+	+	+	+	+	+
13	Un	+	+	+	+	+	+
14	Un	+	+	+	+	+	+
15	Un	+	+	+	+	+	+
16	Un	+	+	+	+	+	+
17	Un	+	+	+	+	+	+
18	Un	+	+	+	+	+	+
19	Un	–	–	+	+	+	+
20	Un	–	–	+	+	+	+
21	Un	–	ND	+	+	+	ND
22	Un	–	–	+	+	+	+
23	Un	–	–	+	+	+	+
24	Un	–	ND	+	ND	+	ND
25	Un	–	–	+	+	+	+
26	Hy	–	–	+	+	+	+
27	Hy	–	–	+	+	+	+
28	Hy	–	–	+	+	+	+
29	Hy	–	–	+	+	+	+
30	Hy	+	+	+	+	+	+

We, western blot analysis; Im, immunostaining; Un, underphosphorylated; Hy, hyperphosphorylated; ND, not done.

tumours to determine which of these cell cycle regulatory proteins affect the phosphorylation of pRB. Cyclin E and Cdk2 were overexpressed in all tumours in comparison with normal

Figure 5 Immunostaining of (A) pRB, (B) P21/Cip1, (C) Cdk2, and (D) cyclin E in breast cancer tissue using serial sections. (A) pRB immunoreactivity is evident in the majority of nuclei ($\times 240$). (B) Almost half of cancer cells were positive for P21/Cip1 ($\times 240$). (C) and (D) Typical staining pattern of Cdk2/Cdc2 (C) and cyclin E (D). Immunoreactive cells are observed in the nuclei and cytoplasm ($\times 240$). The staining pattern of these four proteins in invasive tumour is similar to that of in situ tumour.

mammary ducts and glands, while their expression was not associated with either tumour size or cancer stage. This seems to indicate that the expression of Cdk2 and cyclin E generally occurs early in the carcinogenesis of breast cancer.

The expression of p21/Cip1 has been shown to be induced through both p53 dependent²⁹ and independent pathways.^{30–34} We previously examined the staining patterns of p53 and p21/Cip1 for 104 breast tumours and found that most cases (73%) showed an inverse association between these two proteins.³⁵ In other words, the expression of p21/Cip1 seemed to be induced mainly by the p53 dependent pathway. In the present study, p21/Cip1 was found to stain positively in 19 of the 30 tumours, and pRB was underphosphorylated in 18 of the 19 p21/Cip1 positive tumours. However, four of the five tumours with hyperphosphorylated pRB lacked p21/Cip1 expression. Thus p21/Cip1 showed a statistically significant relation with the phosphorylation of pRB ($p < 0.05$). These results suggest that p21/Cip1 is effective in the suppression of pRB phosphorylation through the inhibition of Cdk2 activity. This phenomenon seems to represent a negative feedback system for cell proliferation control, although the reason why such overexpression of pRB was found in breast cancer remains unclear. On the other hand, there were seven tumours with underphosphorylated pRB in spite of insuffi-

Table 3 Relation between p21/Cip1 expression and pRB phosphorylation

p21/Cip1	Underphosphorylated form of pRB	Hyperphosphorylated form of pRB
Yes	18	1*
No	7	4*
Total	25	5

* $p < 0.05$.

cient p21/Cip1 and sufficient expression of cyclin E and Cdk2. In these cases it has been suggested that other Cdk inhibitors, such as p16 and p27, might play an important role in the inhibition of positive regulators.^{36–37} The results of the present study thus suggest that the phosphorylation of pRB may depend on differences in total activity or in the amounts of negative and positive regulators.

An examination of the relevance of these proteins for prognosis of the disease indicated that no other proteins except pRB were related to prognostic factors. Tumours with underphosphorylated pRB showed a lower frequency of lymph node metastasis ($p < 0.01$), indicating that pRB may be a useful prognostic factor in breast cancer.

In summary, most breast cancers examined showed underphosphorylated pRB in spite of overexpression of cyclin E and Cdk2, and p21/Cip1 may effectively suppress cyclin E/Cdk2 in these tumours. However, further studies are required in relation to cell cycle regulation and to other associated proteins such as Cdk4/

cyclin D and Cdk4 inhibitor p16, because these proteins have also been found to participate in cell cycle regulation.

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